JB
Journals.ASM.org

# Deoxycytidine Deaminase-Deficient *Escherichia coli* Strains Display Acute Sensitivity to Cytidine, Adenosine, and Guanosine and Increased Sensitivity to a Range of Antibiotics, Including Vancomycin

**Tina Manzhu Kang, Jessica Yuan, Alice Zhou, Casey Beppler, Jeffrey H. Miller**

Department of Microbiology, Immunology, and Molecular Genetics and Molecular Biology Institute, University of California, and David Geffen School of Medicine, Los Angeles, California, USA

**We show here that deoxycytidine deaminase (DCD)-deficient mutants of *Escherichia coli* are hypersensitive to killing by exogenous cytidine, adenosine, or guanosine, whereas wild-type cells are not. This hypersensitivity is reversed by exogenous thymidine. The mechanism likely involves the allosteric regulation of ribonucleotide reductase and severe limitations of the dTTP pools, resulting in thymineless death, the phenomenon of cell death due to thymidine starvation. We also report here that DCD-deficient mutants of *E. coli* are more sensitive to a series of different antibiotics, including vancomycin, and we show synergistic killing with the combination of vancomycin and cytidine. One possibility is that a very low, subinhibitory concentration of vancomycin enters Gram-negative cells and that this concentration is potentiated by chromosomal lesions resulting from the thymineless state. A second possibility is that the metabolic imbalance resulting from DCD deficiency affects the assembly of the outer membrane, which normally presents a barrier to drugs such as vancomycin. We consider these findings with regard to ideas of rendering Gram-negative bacteria sensitive to drugs such as vancomycin.**

A crucial step in the biosynthesis of thymidine, one of the components of DNA, is the deamination of one of the forms of deoxycytosine by the enzyme deoxycytidine deaminase (DCD) (1–5). In *Escherichia coli* and certain other Gram-negative bacteria, this enzyme operates at the triphosphate level (on dCTP), deaminating dCTP to dUTP, which is then hydrolyzed to dUMP, as shown in Fig. 1a (e.g., see references 4 and 5), whereas in most Gram-positive microorganisms and in eukaryotes, this enzyme operates at the monophosphate level, deaminating dCMP to dUMP (Fig. 1b) (6–8). In both cases, the key intermediate is dUMP, since this is converted to dTMP by thymidylate synthase. Figure 1 also depicts a second route for the production of thymidine, namely, the conversion of UDP to dUDP by ribonucleotide reductase (RNR). dUDP is converted either to dUTP and subsequently to dUMP or to dUMP directly, depending on the organism (Fig. 1a and b). The UDP pools are generated either by *de novo* synthesis or from the degradation of RNA by polynucleotide phosphorylase (PNP) (9, 10). Mutants lacking DCD have decreased levels of dTTP and increased levels of dCTP (8, 11–14), leading to increased mutation rates (8, 11, 13–15) and, in *Schizosaccharomyces pombe*, to greater sensitivity to some oxidative damage (8). There is a third pathway that can also provide dUMP (16–18), involving the deamination of deoxycytidine (dC) to deoxyuracil (dU) by cytosine deaminase (CDD). dU is then kinased to dUMP. This pathway can apparently alleviate thymidine limitation in DCD-deficient mammalian cell cultures (19), as DCD-deficient mutants require thymidine only if they are also deficient for CDD (12). However, in *E. coli*, the *deoA* gene-encoded thymidine phosphorylase degrades dU, preventing this pathway from supplying thymidine via dUMP generation (see reference 16 and references therein). Inactivation of the *deoA* gene is required to enable this third pathway (16).

In the work described here, we show that DCD-deficient mutants of *E. coli* are hypersensitive to killing by exogenous cytidine, adenosine, or guanosine and that this hypersensitivity is reversed by exogenous thymidine. This is in contrast to previous work with different mammalian cell lines that demonstrated that these lines can be sensitive to deoxyadenosine, deoxyguanosine, and thymidine and that this sensitivity can be relieved by exogenous cytidine or deoxycytidine (20). We discuss these distinctions in light of the allosteric regulation of ribonucleotide reductase. We also report here that DCD-deficient mutants of *E. coli* are more sensitive to a series of different antibiotics, including vancomycin, and consider possible mechanisms for this increased susceptibility.

## MATERIALS AND METHODS

***E. coli* strains.** The DCD-deficient strain used here is from the Keio collection, as described previously by Baba et al. (21), made from the starting strain BW25113 (22). This starting strain (*lacI*$^q$ *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$) was used as the wild type (WT) in the experiments reported here, unless otherwise stated. The *dcd* mutant carries a complete deletion of the *dcd* gene, with a Kan insertion in place of the gene. Strain CAG12099 (23) is MG1655 *zee-3129*::Tn*10*.

**Media.** The following media (24) were used: LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) and minimal medium (minimal medium A) [10.5g $K_2HPO_4$, 4.5 g $KH_2PO_4$, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate · $2H_2O$]. Minimal medium A was the buffer used for dilutions. For growth media, minimal medium A was supplemented with 10 ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 0.5 ml of 1% thiamine hydrochloride (vitamin $B_1$) per liter. Tryptone broth contained 10 g tryptone (1%) and 8 g NaCl per liter (24). The minimal medium A used here, when supplemented with tryptone, contained 1 g tryptone per liter (0.1%).

**Received** 25 November 2013 **Accepted** 9 March 2014
**Published ahead of print** 14 March 2014
Address correspondence to Jeffrey H. Miller, jhmiller@microbio.ucla.edu.

doi:10.1128/JB.01383-13

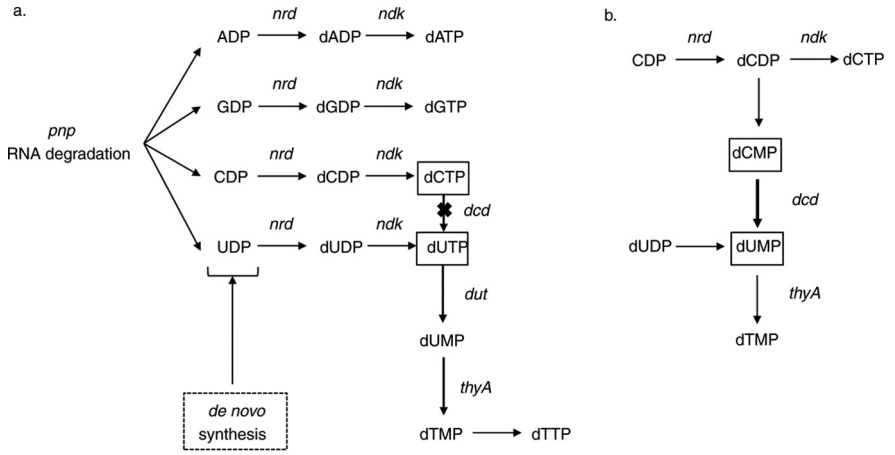


**FIG 1** Metabolic pathway for thymidine production. (a) Thymidine synthesis in *Escherichia coli*. (b) Thymidine synthesis pathways in eukaryotes and Gram-positive bacteria. Note that in *Escherichia coli*, the *dcd*-encoded deoxycytidine deaminase enzyme operates at the triphosphate level (on dCTP), whereas in most Gram-positive microorganisms and in eukaryotes, this enzyme operates at the monophosphate level.

***E. coli* genetic methods.** Unless otherwise stated, all genetic methods were described previously by Miller (24). The *dcd* mutant was purified from single colonies from the Keio collection copy. Experiments were started by inoculating a fresh single colony of the *dcd* mutant. For experiments with LB medium, both the wild type and the *dcd* mutant were grown from single colonies during the day as a seed culture in LB medium supplemented with 50 μg/ml thymidine to a density of $2 \times 10^8$ to $3 \times 10^8$ cells/ml. For antibiotic sensitivity experiments, cultures grown overnight and containing different concentrations of a given antibiotic were seeded with approximately $10^3$ cells by inoculating 2-ml cultures with 50 μl of a $10^{-4}$ dilution of a culture grown during the day. After 18 h of incubation at 37°C on a rotor at 50 rpm, the optical density at 600 nm ($OD_{600}$) was measured. Graphs of these data display percent growth versus that in LB medium and, for synergy experiments, in LB medium supplemented with an antibiotic (vancomycin) and cytidine (see also reference 25). For experiments with minimal medium, the over-day culture in LB medium was spun down, washed, and resuspended in minimal A buffer before diluting the mixture $10^{-4}$ and seeding cultures overnight. For growth curve measurements based on viable cell counts, a culture grown overnight in minimal medium supplemented with 0.1% tryptone and 50 μg/ml thymidine was spun down, washed, and resuspended in minimal buffer. From this suspension, approximately $10^3$ cells were inoculated into different media, and samples were removed at different times and plated directly onto LB medium for viable plate counts. After a 60-min lag time, the viable cell counts showed straight-line growth plots on a semilog plot for several hours.

**Validation controls.** We crossed out the *kan-dcd* insertion/replacement by P1 transduction of a linked Tn*10* marker from strain CAG12099 (23). This restored the wild-type phenotype with respect to colony size and nonsensitivity to exogenous cytidine. We then transduced the *kan-dcd* insertion/replacement from the Keio strain into the starting strain, BW25113, using the linked Tn*10* marker and screening the $Tet^r$ colonies for $Kan^r$. The $Kan^r$ colonies now had a smaller colony size and poor growth in the absence of thymidine and were sensitive to exogenous cytidine.

**Chemicals.** Kanamycin, tetracycline (TET), chloramphenicol (CAM), rifampin (RIF), vancomycin (VAN), nitrofurantoin (NIT), ciprofloxacin (CPR), cephradine (CEP), erythromycin (ERY), adenosine, guanosine, uridine, thymidine, and cytidine were purchased from Sigma (St. Louis, MO).

## RESULTS

**Cytidine, adenosine, and guanosine sensitivity of DCD-deficient mutants in LB medium.** We found that *dcd* strains were strikingly sensitive to cytidine in LB medium and also to adenosine and guanosine at higher concentrations and that these sensitivities can be reversed by the addition of thymidine. Figure 2 exemplifies this, showing cultures grown overnight inoculated with approximately 1,000 cells in broth medium (LB medium) (24) (see Materials and Methods for further details) containing either no additional supplements, 100 μg/ml cytidine, or 100 μg/ml cytidine plus 50 μg/ml thymidine (DCD-deficient mutants grow to somewhat less than full saturation in LB medium not supplemented with additional thymidine [see below]). Concentrations of 20 μg/ml cytidine prevent any detectable growth, and concentrations as low as 10 μg/ml result in 97% growth inhibition (Table 1). In contrast, wild-type *E. coli* can tolerate concentrations of 2,000 μg/ml (Table 2) without evident detrimental effects on growth. In fact, higher concentrations of cytidine resulted in enhanced growth of wild-type strains. (Higher concentrations of thymidine or uridine also resulted in enhanced growth of both wild-type and DCD-deficient strains, with the latter re-

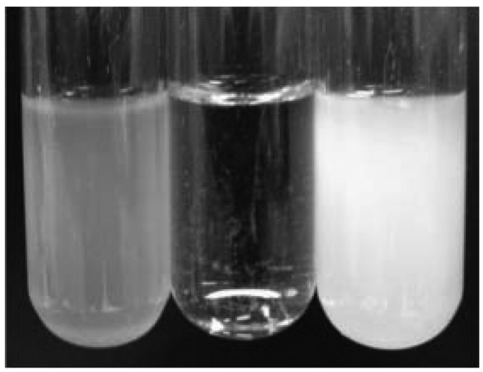


**FIG 2** Growth inhibition of cytidine in the *dcd* mutant and the rescue of killing by thymidine. The *dcd* mutant was grown in LB medium, LB medium with the addition of cytidine, and LB medium with cytidine plus thymidine, at the concentrations shown. Approximately 1,000 cells were inoculated, and growth was monitored after 18 h.

**TABLE 1** Growth effect of cytidine on wild-type and *dcd* mutant strains in LB medium

| Cytidine concn (μg/ml) | Mean % growth ± SD | |
|---|---|---|
| | WT | *dcd* mutant |
| 0 | 100 | 100 |
| 10 | 94.4 ± 8.0 | 2.8 ± 1.0 |
| 20 | 99.6 ± 7.4 | 0.0 ± 0.0 |
| 50 | 99.7 ± 4.1 | 0.0 ± 0.0 |

sponding somewhat more to uridine than the wild type.) DCD-deficient strains were also sensitive to exogenous adenosine and guanosine (Table 2), although in LB medium, higher concentrations were needed to produce the same effect as that with cytidine. For adenosine, 100 μg/ml was required for 90% inhibition of growth and 200 μg/ml was required for 99% inhibition, and for guanosine, 200 μg/ml was required for 83% inhibition and 500 μg/ml was required for 97% inhibition. The rescue of cytidine inhibition by thymidine occurred with as little as 0.15 to 0.25 μg/ml thymidine (Fig. 3). The inhibition/cell killing by adenosine or guanosine was also reversed by exogenous thymidine (data not shown).

**Sensitivity of DCD-deficient mutants in minimal medium.** In minimal medium (see Materials and Methods) supplemented with 0.1% tryptone, DCD-deficient mutants grew poorly but responded to very low levels of thymidine for improved growth. Concentrations of 0.0025 and 0.005 μg/ml thymidine (10 nM and 20 nM, respectively) restored some growth, as judged by saturation levels after 18 h, and a concentration of 0.05 μg/ml restored growth to nearly 80% of the growth with 50 μg/ml (Fig. 4). Growth curves based on viable cell counts (data not shown) (see Materials and Methods) validated these results, as the growth rates with 0.05 μg/ml thymidine were indistinguishable from those with 50 μg/ml (doubling time of 25 min), while the growth rates with no added thymidine were significantly lower (doubling time of 44 min). Cytidine concentrations as low as 1 μg/ml prevented 95% of growth (Table 3). This was easier to see when 0.005 μg/ml thymidine was added, as the growth levels before the addition of cytidine were higher. While 0.005 μg/ml thymidine was sufficient to restore levels of growth nearly 50% or higher under the conditions of the experiments in the absence of cytidine, higher concentrations (0.15 to 0.25 μg/ml thymidine) were required to rescue the cells from cytidine-induced killing (e.g., see Fig. 3). Table 4 shows that adenosine and guanosine have potencies roughly similar to that of cytidine in minimal medium (with 0.1% tryptone broth), even though neither nucleoside is as potent

**TABLE 2** Growth effects of nucleosides on wild-type and *dcd* mutant strains in LB medium

| Nucleoside concn (μg/ml) | Mean $OD_{600}$ ± SD | |
|---|---|---|
| | WT | *dcd* mutant |
| Thymidine | | |
| 0 | 5.50 ± 0.30 | 4.51 ± 0.20 |
| 100 | 5.56 ± 0.24 | 5.75 ± 0.06 |
| 1,000 | 6.38 ± 0.19 | 6.45 ± 0.19 |
| 2,000 | 7.17 ± 0.19 | 6.97 ± 0.11 |
| Cytidine | | |
| 0 | 5.50 ± 0.30 | 4.51 ± 0.20 |
| 100 | 5.92 ± 0.24 | 0.00 ± 0.00 |
| 1,000 | 6.28 ± 0.16 | |
| 2,000 | 6.65 ± 0.11 | |
| Uridine | | |
| 0 | 5.17 ± 0.44 | 3.85 ± 0.21 |
| 100 | 5.13 ± 0.18 | 4.88 ± 0.40 |
| 1,000 | 5.49 ± 0.17 | 6.61 ± 0.17 |
| 2,000 | 6.16 ± 0.23 | 7.50 ± 0.16 |
| Adenosine | | |
| 0 | 4.92 ± 0.21 | 4.01 ± 0.32 |
| 100 | 4.97 ± 0.13 | 0.43 ± 0.21 |
| 200 | 4.91 ± 0.12 | 0.04 ± 0.00 |
| Guanosine | | |
| 0 | 4.92 ± 0.21 | 4.01 ± 0.32 |
| 100 | 4.88 ± 0.09 | 1.74 ± 0.41 |
| 200 | 4.81 ± 0.43 | 0.67 ± 0.39 |
| 500 | 4.74 ± 0.01 | 0.12 ± 0.02 |

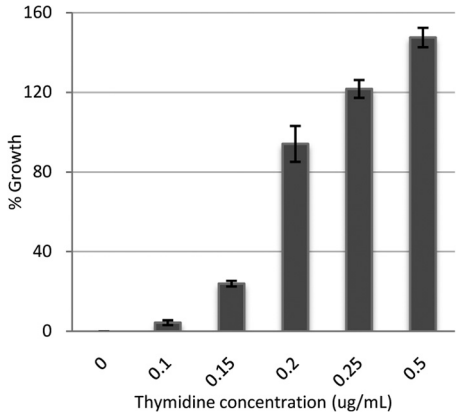


**FIG 3** Thymidine rescues cytidine killing in the *dcd* mutant. The *dcd* mutant was grown in LB medium with 50 μg/ml of cytidine and with various amounts of thymidine (μg/ml). Percent growth was measured against a control in LB medium without added cytidine (or thymidine).

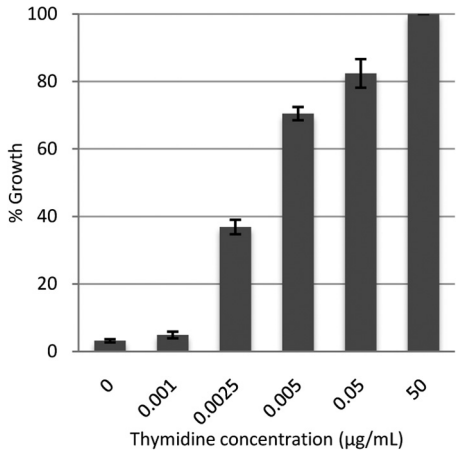


**FIG 4** Growth potentiation of the *dcd* mutant by thymidine in minimal medium. Growth restoration with different, low concentrations of thymidine after 18 h of growth (see Materials and Methods) was measured as a percentage of growth of the culture with 50 μg/ml thymidine.

**TABLE 3** Growth inhibition of wild-type and *dcd* mutant strains by cytidine in minimal medium with added thymidine

| Cytidine concn (μg/ml) | Thymidine concn (μg/ml) | Mean % growth of the *dcd* mutant ± SD |
|---|---|---|
| 0 | 0.005 | 80.1 ± 3.7 |
| 0.5 | 0.005 | 5.1 ± 0.7 |
| 1 | 0.005 | 3.8 ± 0.5 |
| 3 | 0.005 | 3.5 ± 0.4 |
| 0 | 50 | 100 |

an inhibitor as cytidine in LB medium (Table 2). Adenosine is slightly more potent than cytidine in this regard, and guanosine is moderately less potent.

**Addition of cytidine to DCD-deficient strains causes cell killing by thymineless death.** For minimal medium with 0.1% tryptone, viable cell plots show that the addition of cytidine resulted in cell death, as there was a loss of viability after 2 h (Fig. 5). This was not as severe as the rapid death that results in *thyA* strains (e.g., see reference 26) but represents cell death nonetheless.

**DCD-deficient strains show increased sensitivities to a series of antibiotics.** We tested a series of antibiotics, representing different categories of antibiotics, at a range of concentrations in both the wild-type and *dcd* strain backgrounds. DCD-deficient strains are more sensitive to various degrees to each of the antibiotics tested, including ciprofloxacin (CPR), chloramphenicol (CAM), tetracycline (TET), rifampin (RIF), cephradine (CEP), erythromycin (ERY), nitrofurantoin (NIT), and vancomycin (VAN). The concentration giving the largest difference between the wild type and the DCD-deficient derivative is displayed in Fig. 6, and a set of concentrations for vancomycin is shown in Fig. 7. The largest increases in sensitivity were seen for cephradine, nitrofurantoin, and vancomycin. The increased susceptibility to vancomycin is striking. Vancomycin cannot penetrate the outer membrane of Gram-negative bacteria (27), resulting in an MIC of 400 μg/ml (28) (data not shown). Many of the mutants that increase this sensitivity have defects in cell wall or outer membrane synthesis, although some (e.g., *recA* mutants) have defects in DNA recombination or repair functions (29, 30). The fact that *dcd* mutants have an MIC of vancomycin close to 50 μg/ml (Fig. 7) is noteworthy, and we consider this in Discussion, below. Interestingly, the addition of thymidine eliminated the increased sensitivity to vancomycin (data not shown).

**TABLE 4** Growth effect of purines on wild-type and *dcd* mutant strains in minimal medium with the addition of thymidine[a]

| Purine concn (μg/ml) | Mean % growth ± SD | | | |
|---|---|---|---|---|
| | Adenosine | | Guanosine | |
| | WT | *dcd* mutant | WT | *dcd* mutant |
| 0 | 100 | 100 | 100 | 100 |
| 0.1 | | 98.2 ± 9.8 | | |
| 0.5 | | 4.0 ± 0.4 | | |
| 1 | | 2.5 ± 1.0 | | 17.9 ± 1.9 |
| 5 | 95.6 ± 1.3 | 4.5 ± 1.3 | | |
| 10 | 91.6 ± 3.5 | 2.5 ± 0.5 | 83.6 ± 4.2 | 3.3 ± 0.7 |
| 50 | | 0.6 ± 0.7 | 94.5 ± 3.8 | 2.1 ± 1.0 |

[a] A total of 0.005 μg/ml of thymidine was added to the medium in order to supplement growth.

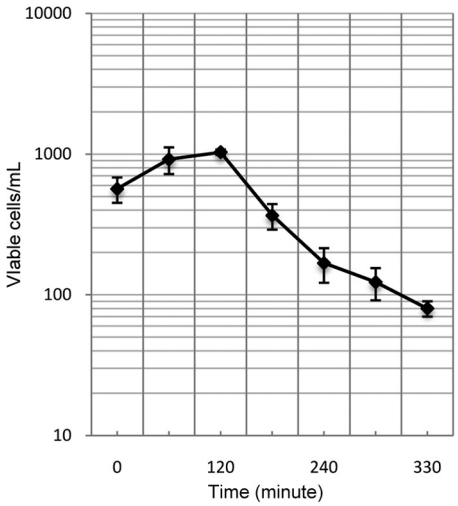


**FIG 5** Loss of viability of the *dcd* mutant in minimal medium with 50 μg/ml of cytidine. Approximately $10^3$ cells were inoculated into different media (see Materials and Methods), and samples were removed at different times and plated directly onto LB medium for viable plate counts.

**Cytidine-mediated killing shows synergy with vancomycin in DCD-deficient strains.** Cytidine-induced killing of *dcd* strains has a synergistic effect with vancomycin (Fig. 8), as the combination of the two treatments results in killing that was significantly greater than the additive effect of each treatment alone (e.g., see reference 25).

## DISCUSSION

The size and balance of the deoxynucleotide triphosphate (dNTP) pools are important for replication fidelity (for a review, see reference 31). Not only do unbalanced pools provoke an increase in

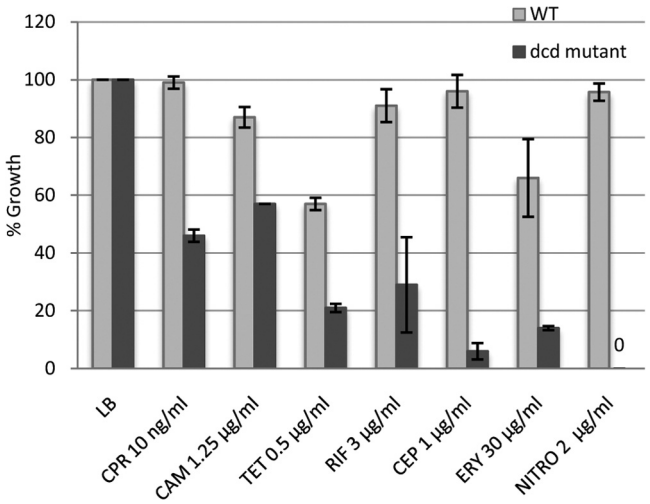


**FIG 6** Increased sensitivity of the *dcd* mutant to various antibiotics. Cultures of the wild type and the *dcd* mutant grown overnight containing different concentrations of a given antibiotic were seeded with approximately $10^3$ cells, and after 18 h of incubation at 37°C, the $OD_{600}$ was measured (see Materials and Methods). The percentage of growth versus the respective strain in LB medium with no antibiotic is shown. NITRO, nitrofurantoin.

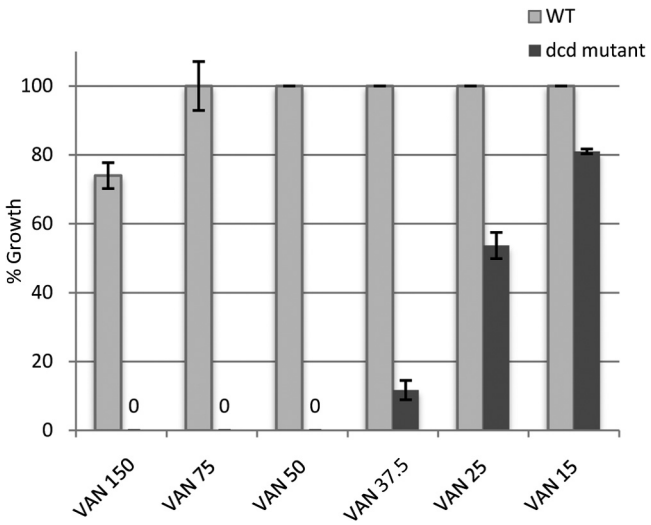


**FIG 7** Increased sensitivity of the *dcd* mutant to vancomycin (μg/ml). Cells were grown with different concentrations of vancomycin (see the legend to Fig. 6). Percent growth is that compared to growth in LB medium alone for each individual strain.

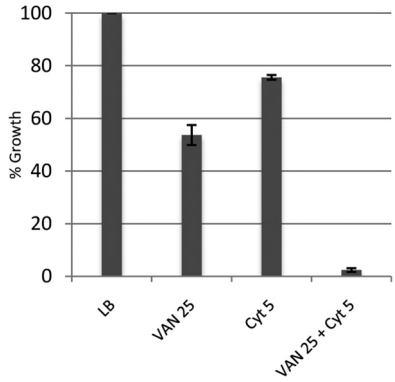


**FIG 8** Synergistic effect of vancomycin with cytidine on the *dcd* mutant. Cells were grown in LB medium or LB medium supplemented with vancomycin (25 μg/ml), cytidine (5 μg/ml), or vancomycin and cytidine. Percent growth is compared to growth in LB medium alone.

mutagenesis (8, 13–15, 31–35), an increase in the total pools of all four dNTPs also leads to increased numbers of mutations (36, 37), and a decrease leads to reduced mutagenesis (38, 39). It is not surprising, therefore, that cells have evolved intricate mechanisms to control the size and balance of dNTP pools. These control mechanisms center around ribonucleotide reductase (RNR), the essential enzyme that generates dNDPs (deoxynucleoside diphosphates) from NDPs (nucleoside diphosphates) (for a review, see reference 40). *E. coli* has 3 different RNRs, each comprised of two different monomer subunits. The principal RNR used during aerobic growth is encoded by the *nrdA* and *nrdB* genes (see reference 40 and references therein). The enzyme has a catalytic site and two separate binding sites for allosteric effectors. One effector binding site modulates the reduction of specific NDPs in response to levels of dGTP, dTTP, and dATP and to the ratio of ATP to dATP. The second site serves as an on-off switch for the overall activity of the enzyme, with the binding of dATP resulting in an inhibition of the total activity. Here, a high ratio of dNTPs to ATP inhibits enzyme activity, where dNTP is dATP, dTTP, or dGTP (e.g., see reference 40). In *E. coli*, this scheme apparently breaks down, however, when DCD is deficient, since the primary route to dTTP is eliminated (Fig. 1 and see below), and the cell must rely on the UDP→dUDP conversion by RNR to ultimately furnish dTTP (Fig. 1A), yet RNR is now partly shut down.

DCD carries out the first step, a deamination, in converting phosphorylated derivatives of deoxycytidine ultimately to deoxythymidine (1–5) (Fig. 1). In *E. coli* and in other gammaproteobacteria, this step occurs at the triphosphate level (e.g., see reference 4 and 5), whereas in most Gram-positive bacteria and in yeast and higher cells, this step occurs at the monophosphate level (6, 7). The direct effect of deleting the *dcd* gene is the buildup of dCTP pools and the decrease of dTTP pools (5, 8, 11–14). As a result, this leads to increased mutagenesis in cell culture (15), yeast (8, 11), and *E. coli* (13); reduced growth rates (e.g., see reference 16); and, in yeast, moderately increased sensitivity to DNA-damaging agents (8). The increased mutagenesis is reduced or eliminated by the addition of thymidine (15) (data not shown). Here, we show that *E. coli dcd* mutants are extremely sensitive to exogenous adenosine, guanosine, or cytidine, in contrast to wild-type *E. coli*. The *dcd* mutants grow slowly in the absence of thymidine, because the primary pathway for thymidine synthesis involves the DCD-catalyzed deamination of dCTP, which is now blocked (Fig. 1a). The second route, via RNR reduction of UDP→dUDP conversion, provides only 20% of the normal amount of thymidine in *E. coli* (see reference 41 and references therein). The addition of even very low levels of thymidine improves growth to near-normal levels (Fig. 4). However, as shown in Tables 1 to 4, the addition of cytidine, adenosine, or guanosine kills *dcd* cells. Thus, it appears that exogenous cytidine, adenosine, or guanosine is ultimately converted to enough dCTP, dATP, or dGTP to sufficiently shut down RNR to the point that an insufficient level of thymidine is provided via the UDP→dUDP pathway. Moreover, de Saint Vincent and coworkers have shown that CTP strongly inhibits UDP reduction by RNR from Chinese hamster cells (12). This shutting down of RNR in a DCD-deficient background leads the cell into thymineless death (Fig. 5), with the latter being a well-studied phenomenon (e.g., see references 26, 42, and 43). A potential third pathway, involving deamination of deoxycytidine (dC) to deoxyuracil (dU) by cytosine deaminase (CDD), is not available in *E. coli* unless the *deoA* gene is inactivated (16, 18), as the *deoA*-encoded enzyme is a deoxycytidine phosphorylase that normally degrades dC and dU (18). In mammalian cell lines, this third pathway is active, so cell lines such as human B lymphoblasts (Raji) that carry a *dcd* mutation are not sensitive to cytosine (19) as they are in *E. coli*, unless they are also deficient in CDD, as is the case with Chinese hamster cell lines (12).

Wild-type *E. coli* is not sensitive to any of the exogenous nucleosides (see, e.g., Table 2) at concentrations as high as $2 \times 10^{-3}$ to $4 \times 10^{-3}$ M. In cultured cells, the expansion of the dATP, dGTP, or dTTP pools results in specific feedback inhibition of RNR (e.g., see reference 40), ultimately resulting in decreased levels of dCTP (20). (This is the inverse of the findings here for *dcd* mutants, in which the expansion of the other dNTP pools limits the dTTP pool.) Thus, different mammalian cell lines have been shown to be sensitive to levels of exogenous adenosine, deoxyadenosine, deoxyguanosine, or thymidine of $10^{-5}$ M to $>10^{-4}$ M (see references 20 and 44 and references therein), and this toxicity can


**FIG 9** Homologous alignment of DCDs among different gammaproteobacteria.

be relieved by exogenous cytidine or deoxycytidine (20). Some mammalian cell culture mutants that are resistant to deoxyadenosine or deoxyguanosine were shown to have altered allosteric regulation of RNR (34, 45, 46), with RNR now responding less to, e.g., dGTP. Other resistant mutants had increased levels of RNR (47), and some mutants resistant to adenosine had hyperactive adenosine deaminase (44), an enzyme that normally reduces the toxic level of adenosine. Certain autoimmune diseases result from an inherited lack of this enzyme (see reference 45).

The increased sensitivity to antibiotics (Fig. 6 and 7) is highlighted by the greatly increased sensitivity to vancomycin, a glycopeptide that cannot normally penetrate the outer membrane of Gram-negative bacteria (27, 48), precluding its use against Gram-negative infections. It is noteworthy that vancomycin acts synergistically with cytidine (Fig. 8). The increased sensitivity to vancomycin is eliminated by the addition of thymidine (data not shown). How can the excess dCTP and the decrease in the dTTP pools result in increased sensitivity to vancomycin? One possibility is that in the partial thymineless state or (in the presence of exogenous cytidine) the full thymineless state, there is a buildup of irreparable chromosomal lesions (43) that act synergistically by potentiating a very low and subinhibitory concentration of vancomycin that penetrates the cell and that itself results in DNA damage. Kohanski and coworkers have proposed and presented evidence for the idea that all bactericidal antibiotics generate hydroxyl radicals that kill the cell by causing DNA damage (49) and that the oxidation of guanine to 8-oxoguanine in the nucleotide pools is a key mechanism by which this occurs (50). An expanded examination of synergistic effects of vancomycin and other antibiotics in a DCD-deficient background might shed light on this model. A second possibility is that the excess dCTP or the decrease in the dTTP pools in a DCD-deficient mutant triggers a metabolic imbalance that in some way affects outer membrane assembly. The phenotype of DCD-deficient mutants with respect to antibiotics is similar to, although less severe than, that seen for SurA-deficient mutants that are greatly affected in outer membrane assembly. SurA is a crucial folding factor/chaperone for beta-barrel proteins (29). If outer membrane assembly is aberrant, proteins such as OmpA, OmpF, LamB, OmpC, and LptD are incorrectly folded and rapidly degraded, which can be tested with antibodies to these proteins by Western blotting. Interestingly, a survey of the Keio collection of gene knockouts in *E. coli* found that of the few mutants showing increased susceptibility to vancomycin, some involved defects in DNA repair or recombination (e.g., *recA* mutants), and others involved defects in outer membrane synthesis (28, 30).

Combinatorial strategies for antibiotic use offer an expanded repertoire of drug therapies (51). Of particular interest are potentiators of existing antibiotics, or "codrugs," a number of which have been used in both laboratory (52, 53) and clinical (54–56) settings. Also, Allison and coworkers demonstrated that specific metabolites can potentiate aminoglycosides acting on *E. coli* and *Staphylococcus aureus* biofilms (57). One desired potentiator would sensitize Gram-negative bacteria to vancomycin. The *dcd* mutants described here have an MIC of vancomycin of 50 μg/ml, suggesting that small-molecule inhibitors of DCD would have the same effect and would be useful potentiators. The three-dimensional (3D) structure of *E. coli* DCD is known (41, 58), facilitating drug design. The *E. coli* DCD enzyme shares extensive homology among Gram-negative bacteria. Figure 9 compares DCD sequences among a set of enzymes from different gammaproteobacteria that are pathogens. The *E. coli* enzyme is 96% identical to individual members of this group, such as *Klebsiella pneumoniae*, with the group as a whole sharing 80% identity. It appears likely that an inhibitor of the *E. coli* enzyme would also inhibit, for instance, the DCD enzyme from *K. pneumoniae*, the leading cause of nosocomial infection among patients in intensive care units (59). Such a potentiator would have a narrow spectrum of action, since DCDs from Gram-positive bacteria and humans have different substrates (dCMP instead of dCTP), and the enzymes from *Bacillus subtilis* and from humans share only weak homology (25% and 26% identities, respectively) to the *E. coli* enzyme. It is highly unlikely that an inhibitor of the *E. coli* enzyme would affect either the Gram-positive population or human cells. It is not clear how many other Gram-negative bacteria outside the gammaproteobacteria use DCD at the triphosphate level and how many use DCD at the monophosphate level, since an examination of the homologies to each of these enzymes from members of different classes of Gram-negative bacteria gave ambiguous results (data not shown). Biochemical experiments are needed to answer this question. Other mutants shown to increase the sensitivity of *E. coli* to vancomycin (*surA* [29] and *smpA* [28] mutants) define targets as well. Also, recently, Morones-Ramirez and coworkers have shown that silver sensitizes *E. coli* to a series of antibiotics, including vancomycin (60), and Hamoud and coworkers reported that

EDTA potentiates the combination of vancomycin plus thymidine (61).

## REFERENCES


1. **McMurry JE, Begley TP.** 2005. The organic chemistry of biological pathways. Roberts and Company, Englewood, NJ.
2. **Neuhard J, Kelin R.** 1996. Chapter II, Biosynthesis and conversions of pyrimidines, p 580–599. *In* Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
3. **Neuhard J, Thomassen E.** 1976. Altered deoxyribonucleotide pools in P2 eductants of *Escherichia coli* K-12 due to a deletion of the *dcd* gene. J. Bacteriol. **126:**999–1001.
4. **O'Donovan GA, Neuhard J.** 1970. Pyrimidine metabolism in microorganisms. Bacteriol. Rev. **34:**278–343.
5. **O'Donovan GA, Edlin G, Fuchs JA, Neuhard J, Thomassen E.** 1971. Deoxycytidine triphosphate deaminase: characterization of an Escherichia coli mutant deficient in the enzyme. J. Bacteriol. **105:**666–672.
6. **Ellims PH, Kao AY, Chabner BA.** 1981. Deoxycytidylate deaminase. Purification and some properties of the enzyme isolated from human spleen. J. Biol. Chem. **256:**6335–6340.
7. **McIntosh EM, Haynes RH.** 1984. Isolation of a *Saccharomyces cerevisiae* mutant strain deficient in deoxycytidine deaminase activity and partial characterization of the enzyme. J. Bacteriol. **158:**644–649.
8. **Sanchez A, Sharma S, Rozenzhak S, Roguev A, Krogan NJ, Chabes A, Russell P.** 2012. Replication fork collapse and genome instability in a deoxycytidylate deaminase mutant. Mol. Cell. Biol. **32:**4445–4454. http://dx.doi.org/10.1128/MCB.01062-12.
9. **Danchin A.** 1997. Comparison between the *Escherichia coli* and *Bacillus subtilis* genomes suggests that a major function of polynucleotide phosphorylase is to synthesize CDP. DNA Res. **4:**9–18. http://dx.doi.org/10.1093/dnares/4.1.9.
10. **Donovan WP, Kushner SR.** 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in Escherichia coli K-12. Proc. Natl. Acad. Sci. U. S. A. **83:**120–124. http://dx.doi.org/10.1073/pnas.83.1.120.
11. **Kohalmi SE, Glattke M, McIntosh EM, Kunz BA.** 1991. Mutational specificity of DNA precursor pool imbalances in yeast arising from deoxycytidylate deaminase deficiency or treatment with thymidylate. J. Mol. Biol. **220:**933–946. http://dx.doi.org/10.1016/0022-2836(91)90364-C.
12. **de Saint Vincent BR, Dechamps M, Buttin G.** 1980. The modulation of the thymidine triphosphate pool of Chinese hamster cells by dCMP deaminase and UDP reductase. Thymidine auxotrophy induced by CTP in dCMP deaminase-deficient lines. J. Biol. Chem. **255:**162–167.
13. **Schaaper R, Mathews CK.** 2013. Mutational consequences of dNTP pool imbalances in *E. coli*. DNA Repair **12:**73–79. http://dx.doi.org/10.1016/j.dnarep.2012.10.011.
14. **Weinberg GL, Ullman B, Martin DW, Jr.** 1981. Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proc. Natl. Acad. Sci. U. S. A. **78:**2447–2451. http://dx.doi.org/10.1073/pnas.78.4.2447.
15. **Weinberg GL, Ullman B, Wright CM, Martin DW, Jr.** 1985. The effects of exogenous thymidine on endogenous deoxynucleotides and mutagenesis in mammalian cells. Somat. Cell Mol. Genet. **11:**413–419. http://dx.doi.org/10.1007/BF01534835.
16. **Weiss B.** 2007. The deoxycytidine pathway for thymidylate synthesis in *Escherichia coli*. J. Bacteriol. **189:**7922–7926. http://dx.doi.org/10.1128/JB.00461-07.
17. **Weiss B.** 2007. YjjG, a dUMP phosphatase, is critical for thymine utilization by *Escherichia coli* K-12. J. Bacteriol. **189:**2186–2189. http://dx.doi.org/10.1128/JB.01645-06.
18. **Weiss B, Wang L.** 1994. De novo synthesis of thymidylate via deoxycytidine in *dcd* (dCTP deaminase) mutants of *Escherichia coli*. J. Bacteriol. **176:**2194–2199.
19. **Perignon J-L, Thullier L, Hamet M, Houlier A-M, Cartier P-H.** 1986. Synergistic toxicity of pyrazofurin and cytidine in cytidine deaminase deficient lymphoid cells (Raji). Int. J. Immunopharmacol. **8:**427–431. http://dx.doi.org/10.1016/0192-0561(86)90127-X.
20. **Dahbo Y, Eriksson S.** 1985. On the mechanism of deoxyribonucleoside toxicity in human T-lymphoblastoid cells. Eur. J. Biochem. **150:**429–434. http://dx.doi.org/10.1111/j.1432-1033.1985.tb09038.x.
21. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006.0008. http://dx.doi.org/10.1038/msb4100050.
22. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
23. **Singer M, Baker TA, Schnitzler G, Deischel SM, Goel M, Dove W, Jaacks KW, Grossman AD, Erickson JW, Gross CA.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
24. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria, p 194–195. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. **Yeh P, Tschumi AI, Kishony R.** 2006. Functional classification of drugs by properties of their pairwise interactions. Nat. Genet. **28:**489–494. http://dx.doi.org/10.1038/ng1755.
26. **Ohkawa T.** 1975. Studies of intracellular thymidine nucleotides. Thymineless death and the recovery after re-addition of thymine in *Escherichia coli* K12. Eur. J. Biochem. **60:**57–66.
27. **Nikaido H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836. http://dx.doi.org/10.1128/AAC.33.11.1831.
28. **Tamae C, Liu A, Kim K, Sitz D, Hong J, Becket E, Bui A, Solaimani P, Tran KP, Yang H, Miller JH.** 2008. Determination of antibiotic hypersensitivity among 4,000 single gene knockout mutants of *Escherichia coli*. J. Bacteriol. **190:**5981–5988. http://dx.doi.org/10.1128/JB.01982-07.
29. **Justice SS, Hunstad DA, Harper JR, Duguay AR, Pinkner JS, Bann J, Frieden C, Silhavy TJ, Hultgren SJ.** 2005. Periplasmic peptidyl prolyl *cis-trans* isomerases are not essential for viability, but SurA is required for pilus biogenesis in *Escherichia coli*. J. Bacteriol. **187:**7680–7686. http://dx.doi.org/10.1128/JB.187.22.7680-7686.2005.
30. **Liu A, Tran L, Becket E, Lee K, Chinn L, Parker E, Tran K, Miller JH.** 2010. Antibiotic sensitivity profiles determined with an Escherichia coli gene knockout collection: generating an antibiotic bar code. Antimicrob. Agents Chemother. **54:**1393–1403. http://dx.doi.org/10.1128/AAC.00906-09.
31. **Kunz BA, Kohalmi SE, Kunkel TA, Mathews CK, McIntosh EM, Reidy JA.** 1994. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. Mutat. Res. **318:**1–64. http://dx.doi.org/10.1016/0165-1110(94)90006-X.
32. **Lu Q, Zhang X, Almaula N, Mathews CK, Inouye M.** 1995. The gene for nucleoside diphosphate kinase functions as a mutator gene in *Escherichia coli*. J. Mol. Biol. **254:**337–341. http://dx.doi.org/10.1006/jmbi.1995.0620.
33. **Meuth M.** 1989. The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. Exp. Cell Res. **181:**305–316. http://dx.doi.org/10.1016/0014-4827(89)90090-6.
34. **Meuth M, Aufreiter E, Reichard P.** 1976. Deoxyribonucleotide pools in mouse-fibroblast cell lines with altered ribonucleotide reductase. Eur. J. Biochem. **71:**39–43. http://dx.doi.org/10.1111/j.1432-1033.1976.tb11087.x.
35. **Miller JH, Funchain P, Clendenin W, Huang T, Nguyen A, Wolff E, Yeung A, Chiang J, Garibyan L, Slupska MM, Yang H.** 2002. *Escherichia coli* strains (*ndk*) lacking nucleoside diphosphate kinase are powerful mutators for base substitutions and frameshifts in mismatch repair deficient strains. Genetics **162:**5–13. http://www.genetics.org/content/162/1/5.long.
36. **Gon S, Napolitano R, Rocha W, Coulon S, Fuchs RP.** 2011. Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced-mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **108:**19311–19316. http://dx.doi.org/10.1073/pnas.1113664108.
37. **Wheeler LJ, Rajagopal I, Mathews CK.** 2005. Stimulation of mutagenesis by proportional deoxynucleoside triphosphate accumulation in *Escherichia coli*. DNA Repair **4:**1450–1456. http://dx.doi.org/10.1016/j.dnarep.2005.09.003.
38. **Becket E, Tse L, Yung M, Cosico A, Miller JH.** 2012. Polynucleotide phosphorylase plays an important role in the generation of spontaneous mutations in *Escherichia coli*. J. Bacteriol. **194:**5613–5620. http://dx.doi.org/10.1128/JB.00962-12.
39. **Laureti L, Selva M, Dairou J, Matic I.** 2013. Reduction of dNTP levels enhances DNA replication fidelity in vivo. DNA Repair **12:**300–305. http://dx.doi.org/10.1016/j.dnarep.2013.01.009.
40. **Hofer A, Crona M, Logan DT, Sjöberg B-M.** 2012. DNA building blocks:

keeping control of manufacture. Crit. Rev. Biochem. Mol. Biol. **47:**50–63. http://dx.doi.org/10.3109/10409238.2011.630372.
41. **Thymark M, Johansson E, Larsen S, Willmoës M.** 2008. Mutational analysis of the nucleotide binding site of Escherichia coli dCTP deaminase. Arch. Biochem. Biophys. **470:**20–26. http://dx.doi.org/10.1016/j.abb.2007.10.013.
42. **Cohen SS, Barner HD.** 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **40:**885–893. http://dx.doi.org/10.1073/pnas.40.10.885.
43. **Kuong KJ, Kuzminov A.** 2010. Stalled replication fork repair and misrepair during thymineless death in *Escherichia coli*. Genes Cells **15:**619–634. http://dx.doi.org/10.1111/j.1365-2443.2010.01405.x.
44. **Fernandez-Mejia C, Debatisse M, Buttin G.** 1984. Adenosine-resistant Chinese hamster fibroblast variants with hyperactive adenosine-deaminase: an analysis of the protection against exogenous adenosine afforded by increased activity of the deamination pathway. J. Cell. Physiol. **120:**321–328. http://dx.doi.org/10.1002/jcp.1041200310.
45. **Ullman B, Gudas LJ, Clift SM, Martin DW, Jr.** 1979. Isolation and characterization of purine-nucleoside phosphorylase-deficient T-lymphoma cells and secondary mutants with altered ribonucleotide reductase: genetic model for immunodeficiency disease. Proc. Natl. Acad. Sci. U. S. A. **76:**1074–1078. http://dx.doi.org/10.1073/pnas.76.3.1074.
46. **Ullman B, Gudas LJ, Caras IW, Erilsson S, Weinberg GL, Wormsted MA, Martin DW, Jr.** 1981. Demonstration of normal and mutant proteins M1 subunits of deoxyGTP-resistant ribonucleotide reductase from mutant mouse lymphoma cells. J. Biol. Chem. **256:**10189–10192.
47. **Meuth M, Green H.** 1974. Alterations leading to increased ribonucleotide reductase in cells selected for resistance to deoxynucleoside. Cell **3:**367–374. http://dx.doi.org/10.1016/0092-8674(74)90052-X.
48. **Pages JM, James CE, Winterhalter M.** 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. **6:**893–903. http://dx.doi.org/10.1038/nrmicro1994.
49. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130:**797–810. http://dx.doi.org/10.1016/j.cell.2007.06.049.
50. **Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC.** 2012. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science **336:**315–319. http://dx.doi.org/10.1126/science.1219192.
51. **Cottarel G, Wierzbowski J.** 2007. Combination drugs, an emerging option for antibacterial therapy. Trends Biotechnol. **25:**547–555. http://dx.doi.org/10.1016/j.tibtech.2007.09.004.
52. **Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho DD, Chamberland S, Renau TT, Ishida H, Lee VJ.** 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. **45:**105–116. http://dx.doi.org/10.1128/AAC.45.1.105-116.2001.
53. **Nelson ML, Levy SB.** 1999. Reversal of tetracycline resistance by different bacterial tetracycline resistance determinants by an inhibitor of the Tet(B) antiport protein. Antimicrob. Agents Chemother. **43:**1719–1724.
54. **Buynak JD.** 2006. Understanding the longevity of the β-lactam antibiotics and of antibiotic/β-lactamase inhibitor combinations. Biochem. Pharmacol. **71:**930–940. http://dx.doi.org/10.1016/j.bcp.2005.11.012.
55. **Hikida M, Kawashima K, Yoshida M, Mitsuhashi S.** 1992. Inactivation of new carbapenem antibiotics by dehydropeptidase-1 from porcine and human renal cortex. J. Antimicrob. Chemother. **30:**129–134. http://dx.doi.org/10.1093/jac/30.2.129.
56. **Hoffman J, Trimble J, Brophy GM.** 2009. Safety of imipenem/cilastin in neurocritical care patients. Neurocrit. Care **10:**403–407. http://dx.doi.org/10.1007/s12028-008-9170-z.
57. **Allison KR, Brynildsen MP, Collins JJ.** 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature **473:**216–220. http://dx.doi.org/10.1038/nature10069.
58. **Johansson E, Fano M, Bynck JH, Neuhard J, Larsen S, Sigurskjold BW, Christensen U, Willemoës M.** 2005. Structures of dCTP deaminase from *Escherichia coli* with bound substrate and product: reaction mechanism and determinants of mono- and bifunctionality for a family of enzymes. J. Biol. Chem. **280:**3051–3059. http://dx.doi.org/10.1074/jbc.M409534200.
59. **Lockhart SR, Abramson MA, Beekmann SE, Gallagher G, Riedel S, Diekema DJ, Quinn JP, Doern GV.** 2007. Antimicrobial resistance among Gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004. J. Clin. Microbiol. **45:**3352–3359. http://dx.doi.org/10.1128/JCM.01284-07.
60. **Morones-Ramirez JR, Winkler JA, Spina CS, Collins JJ.** 2013. Silver enhances antibiotic activity against Gram negative bacteria. Sci. Transl. Med. **5:**190ra81. http://dx.doi.org/10.1126/scitranslmed.3006276.
61. **Hamoud R, Zimmermann S, Reichling J, Wink M.** 18 November 2013. Synergistic interactions in two-drug and three-drug combinations (thymol, EDTA, and vancomycin) against multi drug resistant bacteria including *E. coli*. Phytomedicine [Epub ahead of print.] http://dx.doi.org/10.1016/j.phymed.2013.10.016.